

# 16S rRNA Gene Sequencing, Multilocus Sequence Analysis, and Mass Spectrometry Identification of the Proposed New Species “*Clostridium neonatale*”

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In 2002, an outbreak of necrotizing enterocolitis in a Canadian neonatal intensive care unit was associated with a proposed novel species of *Clostridium*, “*Clostridium neonatale*.” To date, there are no data about the isolation, identification, or clinical significance of this species. Additionally, *C. neonatale* has not been formally classified as a new species, rendering its identification challenging. Indeed, the *C. neonatale* 16S rRNA gene sequence shows high similarity to another *Clostridium* species involved in neonatal necrotizing enterocolitis, *Clostridium butyricum*. By performing a polyphasic study combining phylogenetic analysis (16S rRNA gene sequencing and multilocus sequence analysis) and phenotypic characterization with mass spectrometry, we demonstrated that *C. neonatale* is a new species within the *Clostridium* genus *sensu stricto*, for which we propose the name *Clostridium neonatale* sp. nov. Now that the status of *C. neonatale* has been clarified, matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) can be used for better differential identification of *C. neonatale* and *C. butyricum* clinical isolates. This is necessary to precisely define the role and clinical significance of *C. neonatale*, a species that may have been misidentified and underrepresented during previous neonatal necrotizing enterocolitis studies.

*Clostridium* is a genus of medical importance that includes pathogens responsible for several diseases in adults and children, such as *Clostridium difficile* antibiotic-associated colitis and *Clostridium perfringens* enteric diseases (1, 2). In premature neonates, if clostridia are the most common anaerobes found in fecal samples during the first 2 months of life (3–7), then asymptomatic intestinal carriage of toxigenic or nontoxigenic strains is common and not systematically associated with infections, particularly for *C. difficile* (8, 9). However, various clostridial species have been recovered in neonatal bacteremia (10), and several reports have suggested the role of clostridia in the pathogenesis of necrotizing enterocolitis (NEC) (11–14), a devastating neonatal gastrointestinal disease with high morbidity and mortality rates (15).

In 2002, an outbreak of NEC occurred in six neonates within a 2-month period in a Canadian neonatal intensive care unit (14). Blood cultures from three premature neonates grew the same strain proposed to belong to a novel species of *Clostridium*, “*Clostridium neonatale*,” based on 16S rRNA gene sequencing (16). More recently, *C. neonatale* and *Clostridium butyricum*, another *Clostridium* species involved in NEC, were significantly overrepresented among colonic mucosal samples from premature piglets with NEC (17). Compared to *C. neonatale*, *C. butyricum* has been frequently recovered from biological samples of premature neonates suffering from NEC (18, 19). In quail and chicken animal models of NEC, *C. butyricum* was shown to be responsible for NEC-like lesions (i.e., gas cysts, hemorrhagic lesions, and mucosal necrosis) (20–23).

*C. neonatale* has not been formally classified as a new species, which contributes to its ambiguous identification (5, 24). The confusing status of this species explains the absence of data on its isolation, identification, or clinical significance. As a consequence, misidentification and/or underrepresentation of *C. neonatale* populations during previous NEC studies may have occurred. In the present study, using a polyphasic approach, we investigated

the relationship between *C. neonatale* and *C. butyricum* at the species level. On the basis of a phylogenetic analysis using 16S rRNA gene sequencing and multilocus sequence analysis (MLSA), we show that *C. neonatale* can be considered a new species within the *Clostridium* genus *sensu stricto*. Additionally, the use of mass spectrometry allowed for the differential phenotypic identifications of *C. neonatale* and *C. butyricum* clinical isolates. Although *Clostridium beijerinckii* has not been associated with NEC pathogenesis or isolated from the human gut, it was included in the present study as a species related to *C. butyricum* (25).

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *C. neonatale*, *C. butyricum*, and *C. beijerinckii* strains included in this study are listed in Table 1; the CblF strains were isolated from the feces of premature neonates as previously described (26), and the AIP strains were from the Unité des Bactéries Anaérobies et Toxines (Institut Pasteur, Paris, France). Some of the strains were used only for *in silico* analysis (Table 1, footnote c).

Strains were grown on either Columbia agar medium (Oxoid, Dardilly, France) supplemented with 5% (vol/vol) sheep blood or TGYH broth (30 g/liter tryptone, 5 g/liter glucose, 20 g/liter yeast extract, and 5 mg/liter hemin) and incubated for 24 h at 37°C in an anaerobic chamber (80% N<sub>2</sub>, 10% CO<sub>2</sub>, and 10% H<sub>2</sub>) (AES Chemunex, Bruz, France). Rapid ID 32A

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TABLE 1 Strains used in this study

| Species  | Strain designation <sup>a</sup>                           | Source (reference)                 | GenBank sequence accession no. (status) | Remark                    |
|--|---|------------------------------------|---|---------------------------|
| <i>Clostridium butyricum</i><br>( <i>n</i> = 14) | 1002  | Newborn feces (25)                 |   |                           |
|  | 144-3 <sup>b</sup>  | Newborn feces (25)                 |   |                           |
|  | VPI 3266 <sup>T</sup> (= ATCC 19398 <sup>T</sup> )        | Magot et al. (25)                  | AQQF00000000.1 (assembly)               |                           |
|  | 5521 <sup>c</sup>   |                                    | ABDT00000000 (assembly)                 | BoNT E positive           |
|  | E4 str.BoNT E BL5262 <sup>c</sup>                         |                                    | ACOM00000000 (assembly)                 | BoNT E positive           |
|  | AIP 05.07   | Newborn feces (this study)         |   | Proctorrhagia             |
|  | AIP 224.07  | Newborn feces (this study)         |   |                           |
|  | CblF 7  | Preterm neonate feces (this study) |   |                           |
|  | CblF 9  | Preterm neonate feces (this study) |   |                           |
|  | CblF 17   | Preterm neonate feces (this study) |   |                           |
|  | CblF 20   | Preterm neonate feces (this study) |   |                           |
|  | CblF 43   | Preterm neonate feces (this study) |   |                           |
|  | CblF 47   | Preterm neonate feces (this study) |   |                           |
|  | AIP 86.09   | Newborn feces (this study)         |   | Enterocolitis             |
| <i>Clostridium beijerinckii</i> ( <i>n</i> = 6)  | CNRZ 531  | Dairy products (25)                |   |                           |
|  | VPI 2965  | Magot et al. (25)                  |   |                           |
|  | VPI 2966  | Magot et al. (25)                  |   |                           |
|  | VPI 2983  | Magot et al. (25)                  |   |                           |
|  | NCIMB 8052 <sup>c</sup>                                   |                                    | CP000721 (complete)                     |                           |
| <i>Clostridium neonatale</i><br>( <i>n</i> = 4)  | VPI 5481 <sup>T</sup> (= ATCC 25752 <sup>T</sup> )        |                                    |   |                           |
|  | AIP 13.07   | Newborn feces (this study)         |   |                           |
|  | CblF 22   | Preterm neonate feces (this study) |   |                           |
|  | CblF 51   | Preterm neonate feces (this study) |   |                           |
|  | AIP 250.09 (= NML 99-A-005 (= ATCC BAA-265 = CCUG 46077)) | Preterm neonate feces (14)         |   | Necrotizing enterocolitis |

<sup>a</sup> AIP, collection of the Unité des Bactéries Anaérobies et Toxines (Institut Pasteur, Paris, France); CblF, EA 4065 "Ecosystème Intestinal, Probiotiques, Antibiotiques" collection (Université Paris Descartes, Paris, France); T, type strain.

<sup>b</sup> DNA only available.

<sup>c</sup> *In silico* analysis.

strips (bioMérieux, Marcy l'Etoile, France) were used for biochemical identifications.

**RAPD assay.** DNA purification from 24-h TGYH broth bacterial culture was performed with InstaGene matrix (Bio-Rad, Marnes la Coquette, France) according to the manufacturer's instructions. PCR was carried out using randomly amplified polymorphic DNA (RAPD) analysis beads (GE Healthcare, Vélizy-Villacoublay, France) as previously described (27). Briefly, 10 ng of purified DNA and 25 pm of primer 1 (5'-GGTGC GGGAA-3') or primer 6 (5'-CCCCTCAGCA-3') was used in a total volume of 25 µl. PCR (1 cycle at 95°C for 5 min followed by 45 cycles at 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min) was performed on a GeneAmp PCR System 2700 thermocycler (Life Technologies, Saint Aubin, France).

**16S rRNA gene sequences and MLSA study.** DNA purification from 24-h TGYH broth bacterial culture was performed with InstaGene matrix (Bio-Rad). The 16S rRNA gene sequences were determined as described previously (28) and compared with sequences available in the GenBank database by using the multisequence advanced BLAST comparison software from the National Center for Biotechnology Information (NCBI) (29).

For the MLSA study, genes were selected among housekeeping genes commonly used in studies for *Clostridium* and other bacteria. With *C. butyricum* genomic sequences in an assembly status, for each selected gene, we extracted the corresponding sequence from the complete genome of *C. beijerinckii* strain NCIMB 8052, which was used as the template to locate and retrieve sequences from two *C. butyricum* genomes in an assembly status (Table 1). Six housekeeping genes were selected: *ddl*, *dnaK*, *groEL*, *gyrA*, *rpoB*, and *CTPs* (Table 2). Primer3 software ([http://biotools.umassmed.edu/bioapps/primer3\\_www.cgi](http://biotools.umassmed.edu/bioapps/primer3_www.cgi)) was used to design the primers used for the PCR amplification of an internal fragment of the selected genes (Table 2). PCRs were performed in a final volume of 60 µl containing 0.5 µM concentrations of forward and reverse primers, 200 µM each deoxynucleoside triphosphate (dNTP), 6 µl template DNA, and 3 U *Taq* DNA polymerase (Invitrogen, Saint Aubin, France) in a 1× amplification buffer containing 1.5 mM MgCl<sub>2</sub>. The cycling conditions consisted of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 90 s (35 cycles), and a final extension at 72°C for 10 min. Purified PCR products were sequenced (both strands) (MWG Biotech Roissy CDG, France) with forward and reverse primers. Each housekeeping gene sequence that differed by one or more nucleotides was con-

TABLE 2 Primers used for the *C. neonatale*, *C. butyricum*, and *C. beijerinckii* MLSA study

| Gene         | Function                 | Gene length (bp) | Amplicon length (bp) | Primer name       | PCR/sequencing primer (5' to 3') | Primer position in gene sequence of <i>C. butyricum</i> 5521 (ABDT00000000) | Size (bp) of fragment analyzed (position in <i>C. butyricum</i> 5521) |
|--------------|--------------------------|------------------|----------------------|-------------------|----------------------------------|---|---|
| <i>ddl</i>   | D-Ala-D-Ala ligase       | 906              | 546                  | P1787 (forward)   | AAATYGGAGTTATAATGGGTGGT          | 5–27  | 447 (82–528)  |
|              |                          |                  |                      | P1789 (reverse)   | CAGGACAWGTTATTTTCATCACCTTT       | 550–526   |   |
| <i>dnaK</i>  | DnaK protein (hsp70)     | 1,842            | 1,014                | P1779 (forward)   | GCCATTGTCTTTCACCTTGA             | 584–604   | 459 (712–1,170)   |
|              |                          |                  |                      | P1780 (reverse)   | TGGAACAATATTCACCACAA             | 1,597–1,576   |   |
| <i>groEL</i> | GroEL protein (Hsp60)    | 1,629            | 999                  | P1775 (forward)   | TGGAAGCTGTTTTAGAAAATCCA          | 626–648   | 249 (1,048–1,296)   |
|              |                          |                  |                      | P1776 (reverse)   | ACATTCCGTCCATTCCCATT             | 1,624–1,605   |   |
| <i>gyrA</i>  | DNA gyrase Subunit A     | 2,508            | 793                  | P1777-2 (forward) | GGTGGAAAGGGTATTCAAGC             | 1,582–1,601   | 699 (1,606–2,304)   |
|              |                          |                  |                      | P1777 (reverse)   | TTCTCATTAATGTTACACCCATTG         | 2,374–2,351   |   |
| <i>rpoB</i>  | RNA polymerase subunit B | 3,708            | 1,281                | P1774 (forward)   | AAACATGCAACGTCAGCAG              | 1,983–2,002   | 414 (2,290–2,703)   |
|              |                          |                  |                      | P1773 (reverse)   | AATCTTTGACCACCAATTGAG            | 3,263–3,242   |   |
| <i>CTPs</i>  | CTP synthase             | 1,608            | 467                  | P1853 (forward)   | CCAGGTGGATTTGGTGATAGA            | 1,060–1,080   | 429 (1,081–1,509)   |
|              |                          |                  |                      | P1854 (reverse)   | TGGAATTGTACTGCAACATACC           | 1,526–1,505   |   |

sidered a different allele, and an arbitrary number was assigned. Each unique allelic pattern over all six loci examined was identified as a sequence type (ST). The START2 software (<http://www.pubmlst.org>) (30) was used to obtain concatenated composite sequences from the six gene sequences and to determine the mean G+C content (expressed as mol%). The average frequencies of synonymous substitutions per potential synonymous site (*dS*) and nonsynonymous substitutions per potential nonsynonymous site (*dN*) were calculated with the START2 software to test the degree of selection on a locus.

Sequence alignments and phylogenetic analyses were conducted using the Molecular Evolutionary Genetics Analysis (MEGA) software version 5 (<http://www.megasoftware.net>) (31). Gene trees were constructed with the unweighted-pair group method with arithmetic mean (UPGMA) (16S rRNA sequences) or with the neighbor-joining method (MLSA) using the Kimura two-parameter model (32) and bootstrapping algorithms contained in MEGA software (33).

**Matrix-assisted laser desorption ionization–time of flight mass spectrometry identification.** Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was used for clostridial species identification as previously described (34). Briefly, fresh clostridial colonies grown on Columbia agar medium were deposited onto a target plate (Bruker Daltonics S.A., Wissembourg, France). After being dried, 1 µl of saturated matrix solution containing HCCA (cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was added to each sample. For each isolate, three independent replicate experiments were performed on a MALDI-TOF MS Microflex spectrometer (Bruker Daltonics S.A.). Positive ions were extruded with an accelerating voltage of 20 kV in linear mode. The crude smear spectrum was the sum of 50 shots performed in six different regions for one sample. Spectrum acquisition was performed within an *m/z* range of 3,640 to 19,055 using the Flex control software 3.0 (Bruker Daltonics S.A.). Calibration was performed before each run with the protein calibration standard I (Bruker Daltonics S.A.). *C. butyricum* strain VPI 3266<sup>T</sup> and *C. beijerinckii* strain VPI 5481<sup>T</sup> and the *C. neonatale* NML 99-A-005 reference strain have been added to the Andromas database based on its standardized procedures (35). Numerical data obtained from the spectrometer (peak value and relative intensity for each peak) were analyzed using the Andromas software (version 3.0–2013) (35).

**Antibiotypes.** Antibiotic susceptibility testing of the strains was performed using the disk diffusion method on brucella agar medium supplemented with 5% laked sheep blood and 1 µg of vitamin K1/ml according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (36). Sixteen antibiotic disks (bioMérieux) were used: amoxicillin, amoxicillin-clavulanic acid, piperacillin, piper-

cillin-tazobactam, ertapenem, imipenem, cefoxitin, cefotaxime, clindamycin, tetracycline, tigecycline, chloramphenicol, moxifloxacin, metronidazole, linezolid, and vancomycin.

**Nucleotide sequence accession numbers.** Allele sequences for the six genes in the MLSA study were deposited in the GenBank database under the accession numbers [KF683388](#) to [KF683391](#) and [KM452703](#) to [KM452708](#) (*CTPs* alleles), [KF683392](#) to [KF683396](#) and [KM452709](#) to [KM452712](#) (*ddl* alleles), [KF683397](#) to [KF683400](#) and [KM452713](#) to [KM452718](#) (*dnaK* alleles), [KF683427](#) to [KF683430](#) and [KM452729](#) to [KM452731](#) (*rpoB* alleles), [KF683406](#) to [KF683409](#) and [KM452719](#) to [KM452723](#) (*groEL* alleles), and [KF683410](#) to [KF683417](#) and [KM452724](#) to [KM452728](#) (*gyrA* alleles).

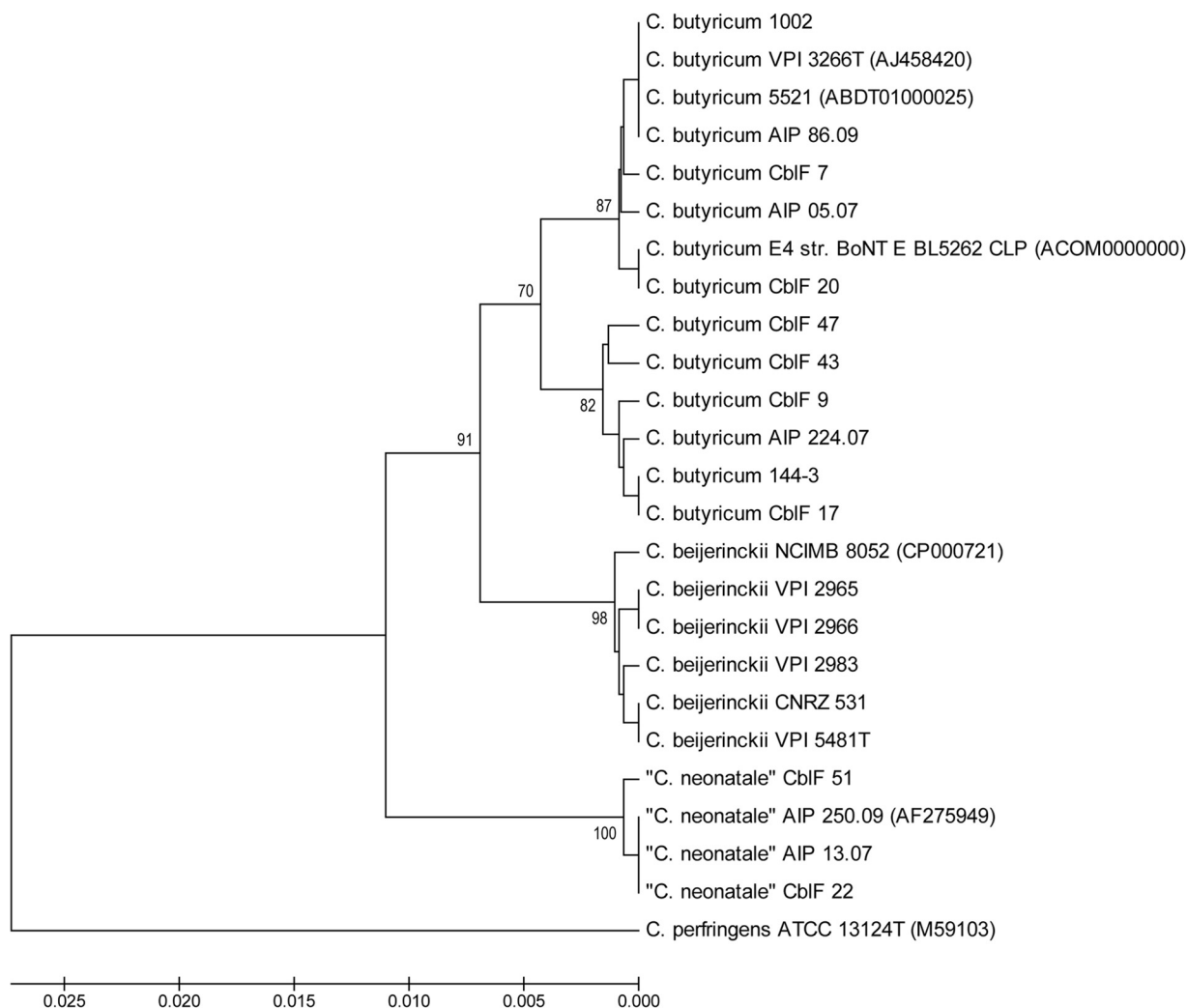
## RESULTS

**Genetic relationships among strains.** RAPD typing was used to prevent the use of redundant isolates and therefore exclude clonally related strains from the study. Based on RAPD data (not shown), 4 *C. neonatale*, 12 *C. butyricum*, and 5 *C. beijerinckii* strains showing different RAPD patterns were included in this study (Table 1).

**16S rRNA gene sequence analysis.** In the 16S rRNA *Clostridium sensu stricto* phylogenetic tree (37), the 16S rRNA sequence of *C. neonatale* (1,406 bp; GenBank accession no. [AF275949](#)) clustered in the branch encompassing *C. butyricum* (type species of the genus), *C. beijerinckii*, *Clostridium saccharobutylicum*, and some other lesser known *Clostridium* species (see Fig. S1 in the supplemental material).

Based on the alignment of 55 16S rRNA gene sequences available in GenBank and corresponding to representatives of *C. butyricum* (*n* = 30), *C. beijerinckii* (*n* = 20), and *C. neonatale* (*n* = 5) strains, we chose a 774-bp shared region (nucleotides 518 to 1291 on *C. butyricum* 5521 16S rRNA gene sequence [GenBank accession number [ABDT01000025](#)]) for the 16S rRNA gene sequence comparison. The built tree presented in Fig. 1 shows that all the strains included in this study were distributed among three nodes representing the three species. In terms of locus variation, 16S rRNA sequence intra- and interspecies similarities ranged from 97.0% to 100.0% and 95.5% to 98.9%, respectively (see Table S2 in the supplemental material).

**MLSA study.** Based on the assumption that genes in the chromosomes of *C. butyricum* and *C. beijerinckii* are syntenic and due



**FIG 1** Partial 16S rRNA sequence (774-bp) relationships among *Clostridium neonatale*, *Clostridium butyricum*, and *Clostridium beijerinckii* strains used in this study. The 774-bp region corresponds to nucleotides 518 to 1291 of the 16S rRNA gene sequence of *Clostridium butyricum* strain 5521 (GenBank accession no. [ABDT01000025](#)). The evolutionary tree was constructed with the MEGA5 software using the UPGMA method and the Kimura 2-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The 16S rRNA sequence from *Clostridium perfringens* ATCC 13124<sup>T</sup> was used as the outgroup in the construction of the tree. Numbers in parentheses are GenBank accession numbers. T, type strain.

to the absence of a complete genomic sequence for *C. butyricum*, we used the *C. beijerinckii* strain NCIMB 8052 genome (GenBank accession number [CP000721](#)) to select the housekeeping genes for our MLSA study. Of the 12 housekeeping genes tested, 6 allowed us to obtain PCR product amplicons for every strain tested. Therefore, the MLSA study was performed with the *ddl*, *dnaK*, *groEL*, *gyrA*, *rpoB*, and *CTPs* genes ([Table 2](#); see also Table S1 in the supplemental material). The MLSA study revealed 18 different sequence types (STs). Genetic variations in the genes investigated ranged from 44 (*groEL*) to 195 (*gyrA*) polymorphic sites, with up to 138 nucleotide differences (1 to 30 and 1 to 138, respectively) (see Table S3 in the supplemental material). The G+C contents of the loci ranged from 31.02% for the *CTPs* locus to 36.04% for the *recA* locus (see Table S3). The calculated nonsynonymous-to-synonymous substitution (*dN/dS*) ratios for the target housekeeping genes ranged from 0.0081 (*rpoB*) to 0.1287 (*CTPs*) (see Table S3). The standardized index of association ( $I^S_A$ ) was 0.7582, reflecting a significant linkage disequilibrium.

To determine the overall divergence of the sequenced fragments of the six loci studied, we spliced these sequences together to obtain an in-frame concatenated composite sequence for each isolate (2,697 bp) and used them for the MLSA study. When calculating the percentage of identity or divergence, in-frame insertions and deletions were not taken into account. Within species, the composite sequence showed high percent similarities, i.e.,  $\geq 96.4\%$  (*C. beijerinckii*),  $\geq 99.8\%$  (*C. neonatale*), and  $\geq 98.4\%$  (*C. butyricum*) (see Table S4 in the supplemental material). Between species, the composite sequence showed low percent similarities ranging from 83.0% to 84.8%, corresponding to high nucleotide differences, i.e., 408 to 411 nucleotides for *C. butyricum* versus *C. neonatale*, 441 to 459 nucleotides for *C. butyricum* versus *C. beijerinckii*, and 417 to 439 nucleotides for *C. beijerinckii* versus *C. neonatale* (see Table S4). An unrooted phylogenetic tree was constructed using the neighbor-joining approach ([Fig. 2](#)). Three clusters with bootstrap values of 100% corresponding to the three species investigated were unambiguously identified, reflecting the

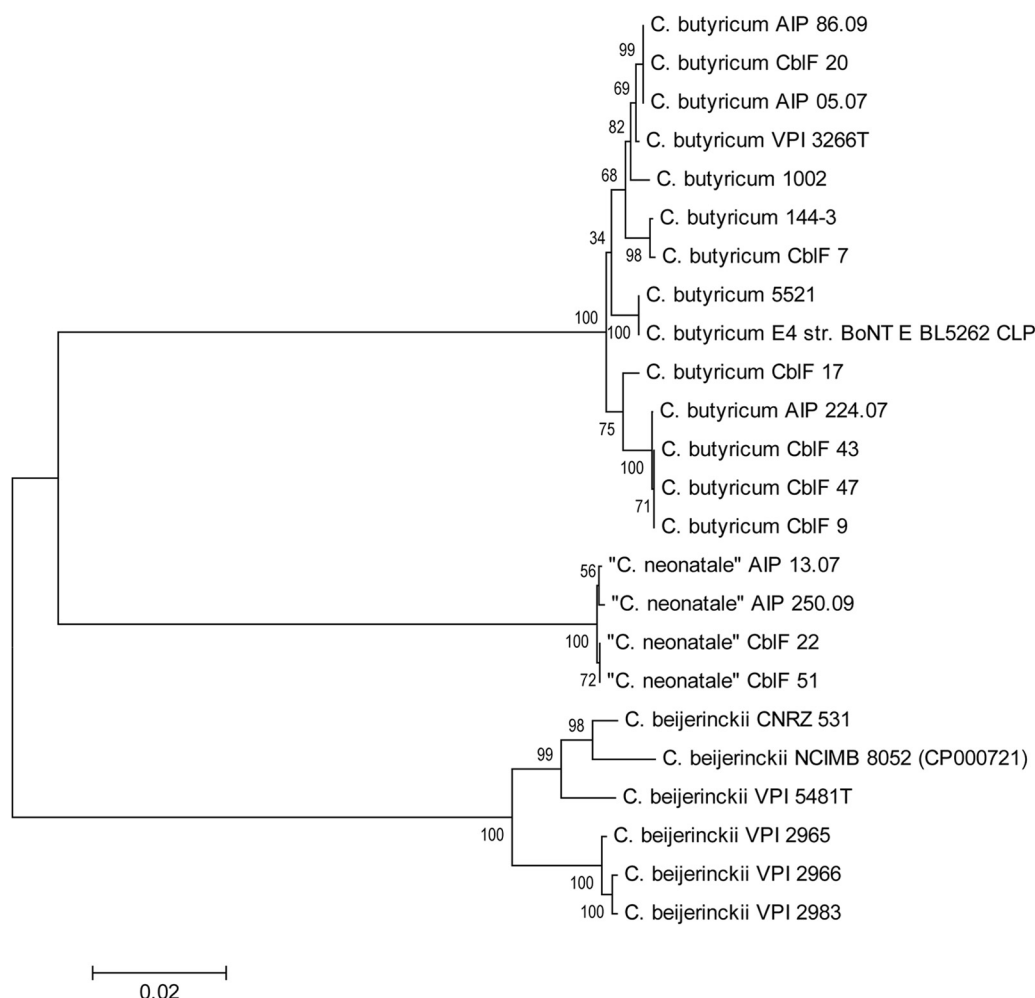


FIG 2 Phylogenetic tree of *Clostridium neonatale*, *Clostridium butyricum*, and *Clostridium beijerinckii* isolates based on the composite sequence of six genes (*CTPs*, *ddl*, *dnaK*, *rpoB*, *groEL*, and *gyrA*) (2,697 bp). The tree was obtained by the neighbor-joining method with the Kimura 2-parameter model. The scale bar indicates the genetic distance. The number shown next to each node indicates the percent bootstrap value of 500 replicates.

high interspecies nucleotide differences observed ( $\geq 408$  over 2,697 nucleotides). For each gene investigated, the topology of the individual tree was similar to that of the tree obtained from concatenated sequences, indicating that recombination events did not occur.

**Phenotypic strain characterization.** The conventional biochemical identification of the isolates included in this study using the rapid ID 32A strips showed that some biochemical differences can be evident among *C. neonatale*, *C. butyricum*, and *C. beijerinckii* strains, but they are not sufficient to allow for a differential identification (see Table S5 in the supplemental material).

*C. butyricum* and *C. beijerinckii* can be identified using a quantitative gas-liquid chromatographic analysis combined with fermentation patterns of glycerol, inositol, and ribose (25, 38). In this study, *C. neonatale* did not show qualitative or quantitative significant differences in its fermentation patterns compared to those of *C. butyricum* (data not shown).

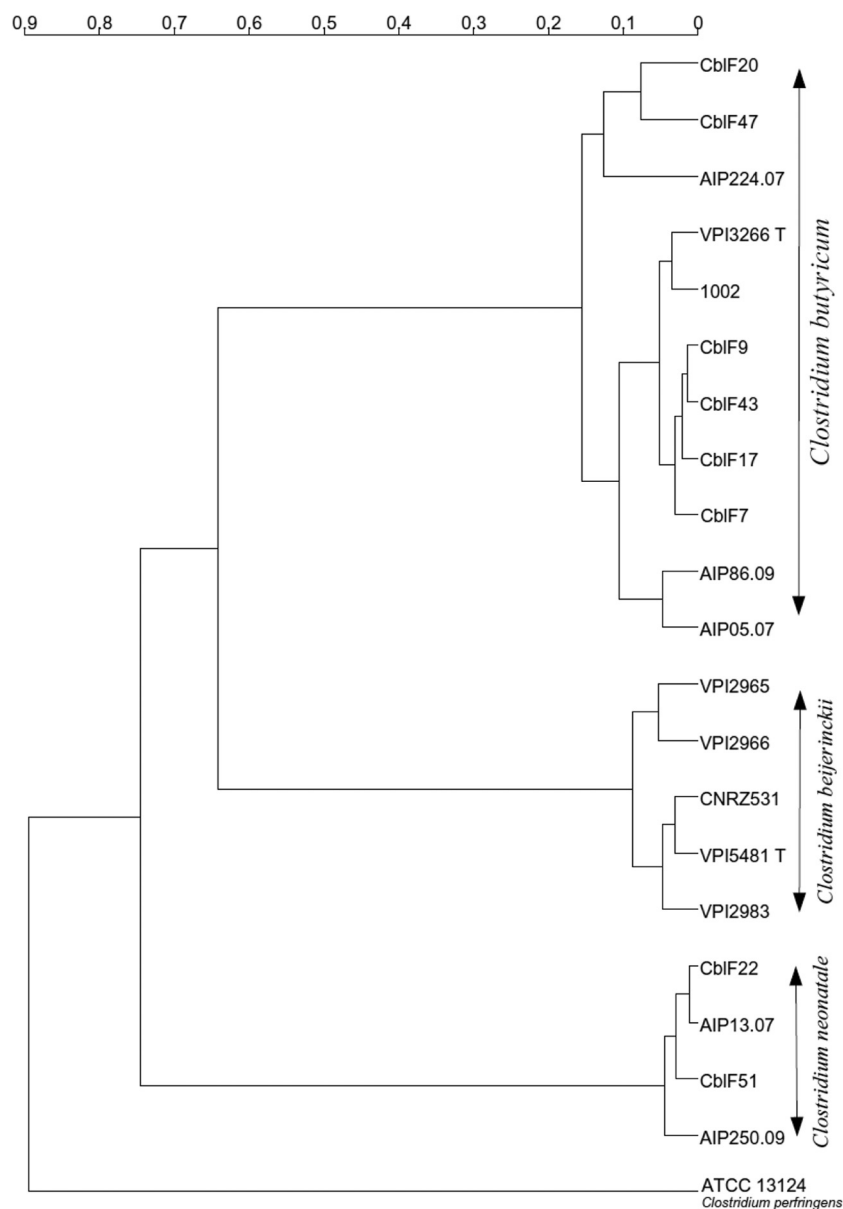
In the present study, we used mass spectrometry for the phenotypic identification of *C. neonatale*. MALDI-TOF spectral mass fingerprints of all the strains are presented in Fig. S2 in the supplemental material. The *C. neonatale* NML 99-A-005 reference

strain and *C. butyricum* VPI 3266<sup>T</sup> and *C. beijerinckii* VPI 5481<sup>T</sup>, used as references for our database, showed distinct mass spectral fingerprints with peak percent differences of 80% between *C. butyricum* and *C. neonatale* or *C. beijerinckii* ( $n = 18$  peaks) and 87% between *C. neonatale* and *C. beijerinckii* ( $n = 16$  peaks). The mass spectra of all the isolates matched the considered species-specific profiles of the database with high similarity percentage values (common peaks) of 100% (*C. neonatale*), 90% to 100% (*C. butyricum*), and 88% to 100% (*C. beijerinckii*). As shown in Fig. 3, the dendrogram constructed from mass profiles shows a graphical representation of the spectral dissimilarities, allowing a clear distribution of all the strains tested into the *C. neonatale*, *C. butyricum*, and *C. beijerinckii* clusters.

In terms of antibiotic resistance profiles, all the *C. neonatale* strains included in this study were susceptible to the tested molecules except for clindamycin, to which the four tested strains were resistant (inhibition zone diameter,  $< 15$  mm).

## DISCUSSION

This study clarifies *C. neonatale* NEC-associated species status within the *Clostridium* genus *sensu stricto*. This was necessary to



**FIG 3** Example of a tree showing *Clostridium neonatale*, *Clostridium butyricum*, and *Clostridium beijerinckii* strains based on spectral fingerprints obtained from whole colonies (crude smear preparation). The tree was obtained using the Andromas software (version 3.0-2013). The scale bar indicates dissimilarity between two spectra based on the Dice coefficient. The *Clostridium perfringens* ATCC 13124 type strain was used as the outgroup in the construction of the tree.

allow for correct clinical differential identification between *C. neonatale* and *C. butyricum*, both of which are *Clostridium* species involved in NEC.

In this study, we first addressed isolate clonality using RAPD in order to present data based on independent strains. The 16S rRNA gene sequence of the *C. neonatale* reference strain clustered in the 16S rRNA *Clostridium sensu stricto* phylogenetic tree (i.e., cluster I according to Collins et al. [37]), which includes the type species of the genus, *C. butyricum* VPI 3266<sup>T</sup>. However, the 16S rRNA gene sequence interspecies percent similarity ranges did not allow applying either the 97% or the 98.7% to 99% limit classically proposed for species delineation within a genus (39). Moreover, 16S rRNA gene sequencing alone is not a sufficient criterion for species delineation and needs to be completed with another genetic

approach such as MLSA, which is considered an excellent alternative for new species delineation (39). Therefore, we performed MLSA to study the phylogenetic relationships among the *C. neonatale*, *C. butyricum*, and *C. beijerinckii* species. Our data indicate a limited contribution of environmental selection to the sequence variation of the six housekeeping genes used for the analysis (low  $dN/dS$  ratios). The standardized index of association,  $I^S_A$ , reflected significant linkage disequilibrium and thus a clonal evolution of *C. neonatale*, *C. butyricum*, and *C. beijerinckii*, as described in *C. difficile* (40). Our MLSA study placed each of the isolates tested in its nominal species-specific group. Altogether, these results, based on two different genetic analyses (16S rRNA and MLSA), give robust and reliable information for considering *C. neonatale* a new species within the *Clostridium* genus *sensu stricto*.

Alfa et al. (14) originally identified their clinical isolates as *Clostridium clostridioforme* by anaerobic identification strips and subsequently proposed that they belong to the *C. neonatale* species based on 16S rRNA gene sequencing. In this study, although conventional techniques showed some differences among the *C. neonatale*, *C. butyricum*, and *C. beijerinckii* strains, they were not sufficiently discriminant to provide a definitive identification. Therefore, we used MALDI-TOF MS as an alternative approach for phenotypic characterizations. Indeed, MALDI-TOF MS has been used for the phenotypic identification of anaerobic bacteria in clinical microbiology with superior resolution and discrimination power compared to those of conventional identification approaches (41). In the present study, MALDI-TOF MS allowed for correct differential identification of *C. neonatale*, *C. butyricum*, and *C. beijerinckii* strains at the species level. These data, together with our genetic analysis, support the idea that *C. neonatale* is a new species within the *Clostridium* genus *sensu stricto*.

In terms of identification of medically important anaerobic bacteria, 16S rRNA sequencing has been reported to confidently identify 62% (full 16S rRNA sequence) and 68% (527-bp 16S rRNA) of these bacteria to the species level (42). When considering only Gram-positive anaerobic bacteria, and particularly *Clostridium* spp., these percentages fall to 50% and 55%, respectively (42 *Clostridium* species tested). In the present study, we used a 774-bp region of the 16S rRNA gene sequence of independent clinical isolates, allowing for distribution of the tested strains among the *C. butyricum*, *C. neonatale*, and *C. beijerinckii* species. Until now, the limitations of *C. neonatale* identification resided in the absence of a formal species classification. Indeed, the absence of a defined type strain would not systematically result in a positive match when using identification software and databases. For instance, the NCBI BLASTN program would not identify the *C. neonatale* NML 99-A-005 16S rRNA gene sequence when using the 16S rRNA sequences (bacteria and archaea) database (on the basis of the last time we accessed it [February 2014]). Another example is the <sup>16</sup>S-BIBI (Bio Informatic Bacteria Identification) software (<http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi> [last accessed February 2014]), which would identify the *C. neonatale* NML 99-A-005 16S rRNA gene sequence when the procaryota\_SSU-rRNA-16S\_lax database is used instead of the more stringent procaryota\_SSU-rRNA-16S\_TS\_stringent database, which gives a NO\_ACCEPTABLE\_CANDIDATE\_VALUE. In this study, we validated MALDI-TOF MS as a cheap, fast, and reliable alternative method for the routine identification of *C. neonatale*. As for 16S rRNA sequencing, MALDI-TOF MS identification is limited by the degree of completeness of the databases, and because *C. neonatale* has not been formally defined as a species, it is not included in the available databases of recently published studies (43, 44).

To date, there is no information about the clinical significance or virulence factors of *C. neonatale*. We had access to some of the clinical data concerning the premature neonates from whom the *C. neonatale* strains were recovered (gestational age, mode of delivery, mode of feeding, and neonatal antibiotic courses), but no specific determinant was associated with *C. neonatale* isolation. *C. neonatale* was susceptible to common anaerobe antimicrobial agents and showed resistance to clindamycin. Although no information is available about *C. neonatale* virulence factors, in animal experimental models, butyrate and gas production were proposed as virulence determinants involved in *C. butyricum*-dependent

NEC lesions (20–23). Since *C. neonatale* and *C. butyricum* strains synthesize similar amounts of butyrate *in vitro*, we hypothesized that *C. neonatale* NEC pathogenesis is also related to fermentation end products, as suggested by Alfa et al. (14). Interestingly, *C. beijerinckii*, related to *C. butyricum*, is not found in neonates with or without NEC, suggesting that the ecological environment of the digestive tract is not advantageous for *C. beijerinckii*.

In conclusion, by performing a polyphasic study combining genetic and phenotypic data, we demonstrated that *C. neonatale* is a new species within the *Clostridium* genus *sensu stricto*, for which we propose the name *Clostridium neonatale* sp. nov. Now that *C. neonatale* status has been clarified, MALDI-TOF MS will be used for better differential identification of *C. neonatale* and *C. butyricum* clinical isolates. Consequently, it will allow the characterization of *C. neonatale* isolates at the clinical level, particularly when considering NEC studies.

## REFERENCES

1. Rupnik M, Wilcox MH, Gerding DN. 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nat. Rev. Microbiol.* 7:526–536. <http://dx.doi.org/10.1038/nrmicro2164>.
2. Mallozzi M, Viswanathan VK, Vedantam G. 2010. Spore-forming bacilli and clostridia in human disease. *Future Microbiol.* 5:1109–1123. <http://dx.doi.org/10.2217/fmb.10.60>.
3. Campeotto F, Suau A, Kapel N, Magne F, Viallon V, Ferraris L, Waligora-Dupriet AJ, Soulaïnes P, Leroux B, Kalach N, Dupont C, Butel MJ. 2011. A fermented formula in pre-term infants: clinical tolerance, gut microbiota, down-regulation of faecal calprotectin and up-regulation of faecal secretory IgA. *Br. J. Nutr.* 105:1843–1851. <http://dx.doi.org/10.1017/S0007114510005702>.
4. Rougé C, Piloquet H, Butel MJ, Berger B, Rochat F, Ferraris L, Des RC, Legrand A, de la Cochetière MF, N'Guyen JM, Vodovar M, Voyer M, Darmaun D, Roze JC. 2009. Oral supplementation with probiotics in very-low-birth-weight preterm infants: a randomized, double-blind, placebo-controlled trial. *Am. J. Clin. Nutr.* 89:1828–1835. <http://dx.doi.org/10.3945/ajcn.2008.26919>.
5. Rougé C, Goldenberg O, Ferraris L, Berger B, Rochat F, Legrand A, Gobel UB, Vodovar M, Voyer M, Roze JC, Darmaun D, Piloquet H, Butel MJ, de la Cochetière MF. 2010. Investigation of the intestinal microbiota in preterm infants using different methods. *Anaerobe* 16:362–370. <http://dx.doi.org/10.1016/j.anaerobe.2010.06.002>.
6. Magne F, Abely M, Boyer F, Morville P, Pochart P, Suau A. 2006. Low species diversity and high interindividual variability in faeces of preterm infants as revealed by sequences of 16S rRNA genes and PCR-temporal temperature gradient gel electrophoresis profiles. *FEMS Microbiol. Ecol.* 57:128–138. <http://dx.doi.org/10.1111/j.1574-6941.2006.00097.x>.
7. Jacquot A, Neveu D, Aujoulat F, Mercier G, Marchand H, Jumas-Bilak E, Picaud JC. 2011. Dynamics and clinical evolution of bacterial gut microflora in extremely premature patients. *J. Pediatr.* 158:390–396. <http://dx.doi.org/10.1016/j.jpeds.2010.09.007>.
8. Enoch DA, Butler MJ, Pai S, Aliyu SH, Karas JA. 2011. *Clostridium difficile* in children: colonisation and disease. *J. Infect.* 63:105–113. <http://dx.doi.org/10.1016/j.jinf.2011.05.016>.
9. Rousseau C, Lemee L, Le MA, Poilane I, Pons JL, Collignon A. 2011. Prevalence and diversity of *Clostridium difficile* strains in infants. *J. Med. Microbiol.* 60:1112–1118. <http://dx.doi.org/10.1099/jmm.0.029736-0>.
10. Brook I. 2010. The role of anaerobic bacteria in bacteremia. *Anaerobe* 16:183–189. <http://dx.doi.org/10.1016/j.anaerobe.2009.12.001>.
11. Kosloske AM, Ulrich JA, Hoffman H. 1978. Fulminant necrotizing enterocolitis associated with clostridia. *Lancet* ii:1014–1016.
12. Cashore WJ, Peter G, Lauermann M, Stonestreet BS, Oh W. 1981. Clostridia colonization and clostridia toxin in neonatal necrotizing enterocolitis. *J. Pediatr.* 98:308–311. [http://dx.doi.org/10.1016/S0022-3476\(81\)80667-1](http://dx.doi.org/10.1016/S0022-3476(81)80667-1).
13. Sturm R, Stanek JL, Stauffer LR, Neblett WW, III. 1980. Neonatal necrotizing enterocolitis associated with penicillin-resistant, toxigenic *Clostridium butyricum*. *Pediatrics* 66:928–931.
14. Alfa MJ, Robson D, Davi M, Bernard K, Van Caeseele P, Harding GK. 2002. An outbreak of necrotizing enterocolitis associated with a novel

- Clostridium* species in a neonatal intensive care unit. Clin. Infect. Dis. 35:S101–S105. <http://dx.doi.org/10.1086/341929>.
15. Neu J, Walker WA. 2011. Necrotizing enterocolitis. N. Engl. J. Med. 364:255–264. <http://dx.doi.org/10.1056/NEJMra1005408>.
  16. Bernard KA, Shuttleworth L, Munro C. 2000. Novel *Clostridium* species derived from neonates with necrotizing enterocolitis, p 192. Final Program Anaerobe 2000 Congress, Manchester, United Kingdom.
  17. Azcarate-Peril MA, Foster DM, Cadenas MB, Stone MR, Jacobi SK, Stauffer SH, Pease A, Gookin JL. 2011. Acute necrotizing enterocolitis of preterm piglets is characterized by dysbiosis of ileal mucosa-associated bacteria. Gut Microbes 2:234–243. <http://dx.doi.org/10.4161/gmic.2.4.16332>.
  18. Kliegman RM, Fanaroff AA. 1984. Necrotizing enterocolitis. N. Engl. J. Med. 310:1093–1103. <http://dx.doi.org/10.1056/NEJM198404263101707>.
  19. Smith B, Bode S, Petersen BL, Jensen TK, Pipper C, Kloppenborg J, Boye M, Krogfelt KA, Molbak L. 2011. Community analysis of bacteria colonizing intestinal tissue of neonates with necrotizing enterocolitis. BMC Microbiol. 11:73. <http://dx.doi.org/10.1186/1471-2180-11-73>.
  20. Popoff MR, Szlyt O, Ravisse P, Dabard J, Ohayon H. 1985. Experimental cecitis in gnotoxenic chickens monoassociated with *Clostridium butyricum* strains isolated from patients with neonatal necrotizing enterocolitis. Infect. Immun. 47:697–703.
  21. Bousseboua H, Le Coz Y, Dabard J, Szlyt O, Raibaud P, Popoff MR, Ravisse P. 1989. Experimental cecitis in gnotobiotic quails monoassociated with *Clostridium butyricum* strains isolated from patients with neonatal necrotizing enterocolitis and from healthy newborns. Infect. Immun. 57:932–936.
  22. Butel MJ, Roland N, Hibert A, Popot F, Favre A, Tessède AC, Bensaada M, Rimbault A, Szlyt O. 1998. Clostridial pathogenicity in experimental necrotizing enterocolitis in gnotobiotic quails and protective role of bifidobacteria. J. Med. Microbiol. 47:391–399. <http://dx.doi.org/10.1099/00222615-47-5-391>.
  23. Waligora-Dupriet AJ, Dugay A, Auzeil N, Huerre M, Butel MJ. 2005. Evidence for clostridial implication in experimental necrotizing enterocolitis through bacterial fermentation in a gnotobiotic quail model. Pediatr. Res. 58:629–635. <http://dx.doi.org/10.1203/01.PDR.0000180538.13142.84>.
  24. Roudière L, Jacquot A, Marchandin H, Aujoulat F, Devine R, Zorogniotti I, Jean-Pierre H, Picaud JC, Jumas-Bilak E. 2009. Optimized PCR-temporal temperature gel electrophoresis compared to cultivation to assess diversity of gut microbiota in neonates. J. Microbiol. Methods 79: 156–165. <http://dx.doi.org/10.1016/j.mimet.2009.08.005>.
  25. Magot M, Carlier JP, Popoff MR. 1983. Identification of *Clostridium butyricum* and *Clostridium beijerinckii* by gas-liquid chromatography and sugar fermentation: correlation with DNA homologies and electrophoretic patterns. J. Gen. Microbiol. 129:2837–2845.
  26. Ferraris L, Butel MJ, Campeotto F, Vodovar M, Roze JC, Aires J. 2012. Clostridia in premature neonates' gut: incidence, antibiotic susceptibility, and perinatal determinants influencing colonization. PLoS One 7:e30594. <http://dx.doi.org/10.1371/journal.pone.0030594>.
  27. Wang X, Maegawa T, Karasawa T, Kozaki S, Tsukamoto K, Gyobu Y, Yamakawa K, Oguma K, Sakaguchi Y, Nakamura S. 2000. Genetic analysis of type E botulinum toxin-producing *Clostridium butyricum* strains. Appl. Environ. Microbiol. 66:4992–4997. <http://dx.doi.org/10.1128/AEM.66.11.4992-4997.2000>.
  28. Carlier JP, K'ouas G, Bonne I, Lozniewski A, Mory F. 2004. *Oribacterium sinus* gen. nov., sp. nov., within the family "Lachnospiraceae" (phylum Firmicutes). Int. J. Syst. Evol. Microbiol. 54:1611–1615. <http://dx.doi.org/10.1099/ijs.0.63060-0>.
  29. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402. <http://dx.doi.org/10.1093/nar/25.17.3389>.
  30. Jolley KA, Feil EJ, Chan MS, Maiden MC. 2001. Sequence type analysis and recombinational tests (START). Bioinformatics 17:1230–1231. <http://dx.doi.org/10.1093/bioinformatics/17.12.1230>.
  31. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731–2739. <http://dx.doi.org/10.1093/molbev/msr121>.
  32. Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111–120. <http://dx.doi.org/10.1007/BF01731581>.
  33. Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics 17:1244–1245. <http://dx.doi.org/10.1093/bioinformatics/17.12.1244>.
  34. Degand N, Carbonnelle E, Dauphin B, Beretti JL, Le BM, Sermet-Gaudelus I, Segonds C, Berche P, Nassif X, Ferroni A. 2008. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of nonfermenting gram-negative bacilli isolated from cystic fibrosis patients. J. Clin. Microbiol. 46:3361–3367. <http://dx.doi.org/10.1128/JCM.00569-08>.
  35. Farfour E, Leto J, Barritault M, Barberis C, Meyer J, Dauphin B, Le Guern AS, Lefleche A, Badell E, Guiso N, Leclercq A, Le MA, Lecuit M, Rodriguez-Nava V, Bergeron E, Raymond J, Vimont S, Bille E, Carbonnelle E, Guet-Revillet H, Lecuyer H, Beretti JL, Vay C, Berche P, Ferroni A, Nassif X, Join-Lambert O. 2012. Evaluation of the Andromas matrix-assisted laser desorption ionization-time of flight mass spectrometry system for identification of aerobically growing Gram-positive bacilli. J. Clin. Microbiol. 50:2702–2707. <http://dx.doi.org/10.1128/JCM.00368-12>.
  36. Comité de l'Antibiogramme de la Société Française de Microbiologie (CASFM). 2012. Bulletin de la Société Française de Microbiologie. [http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM\\_2012.pdf](http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM_2012.pdf).
  37. Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JA. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44:812–826. <http://dx.doi.org/10.1099/00207713-44-4-812>.
  38. Carlier JP. 2002. The metabolic end-products—a rapid identification tool for common anaerobic bacteria isolated in clinical microbiology. Recent Res. Dev. Microbiol. 6:157–175.
  39. Figueras MJ, Beaz-Higalco R, Collado L, Martinez-Murcia A. 2011. Recommendations for new bacterial species description based on analyses of the unrelated genera *Aeromonas* and *Arcobacter*. Bull. BISMIS 2:1–16.
  40. Lemee L, Dhalluin A, Pestel-Caron M, Lemeland JF, Pons JL. 2004. Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types. J. Clin. Microbiol. 42:2609–2617. <http://dx.doi.org/10.1128/JCM.42.6.2609-2617.2004>.
  41. Biswas S, Rolain JM. 2013. Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. J. Microbiol. Methods 92:14–24. <http://dx.doi.org/10.1016/j.mimet.2012.10.014>.
  42. Woo PC, Chung LM, Teng JL, Tse H, Pang SS, Lau VY, Wong VW, Kam KL, Lau SK, Yuen KY. 2007. *In silico* analysis of 16S ribosomal RNA gene sequencing-based methods for identification of medically important anaerobic bacteria. J. Clin. Pathol. 60:576–579. <http://dx.doi.org/10.1136/jcp.2006.038653>.
  43. Veloo AC, Welling GW, Degener JE. 2011. The identification of anaerobic bacteria using MALDI-TOF MS. Anaerobe 17:211–212. <http://dx.doi.org/10.1016/j.anaerobe.2011.03.026>.
  44. Justesen US, Holm A, Knudsen E, Andersen LB, Jensen TG, Kemp M, Skov MN, Gahrn-Hansen B, Møller JK. 2011. Species identification of clinical isolates of anaerobic bacteria: a comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry systems. J. Clin. Microbiol. 49:4314–4318. <http://dx.doi.org/10.1128/JCM.05788-11>.